PATHOGEN DETECTION USING HEADSPACE ANALYSIS[‡]

Christina M. Rudzinski, Rachel Herzig-Marx, Jonathan Lin, Adam Szpiro, Bernadette Johnson

MIT Lincoln Laboratory
244 Wood Street
Lexington, MA 02420

ABSTRACT

The ability to rapidly screen samples for the presence of pathogens offers many advantages. From a security standpoint, the rapid classification of samples as pathogenic or non-pathogenic (suspicious powders, for example) can elicit a more rapid first response and can minimize the labor intensive processing of countless samples in the laboratory, thus saving both time and money. In a clinical setting, pre-symptomatic diagnosis of infection can prevent the onset of life-threatening illnesses, speed up treatment of infection, and eliminate the unnecessary or inappropriate prescription of antibiotics.

The variation in volatile metabolic byproducts produced by bacteria provides a signature with which to identify infectious agents. Analysis of the volatile constituents affords an orthogonal means of identification that can be performed in conjunction with the commonly used PCR and antibody tests. Our research has focused on optimizing and simplifying headspace collection techniques to enable reliable sampling of volatile compounds from both liquid and solid samples. We have also established non-invasive breath-sampling techniques that will allow individuals to be monitored for pre-symptomatic diagnosis of infection. Using thermodesorption gas chromatography-mass spectrometry (GC-MS), the gas-phase signatures of potential threat-agent bacteria and causative agents of respiratory infections have been examined under a variety of conditions. Initial results from this study indicate that headspace signatures provide a means of detection and discrimination that can be applied toward the development of small portable sensors for use in either field or clinical settings.

1. INTRODUCTION

Many sensors are being designed to meet the need for pathogen detection, in particular, to address the threat of biowarfare attacks. Requirements for sensor functionality are broad and are dependant on the situation in which they will be utilized. Likewise, the processing of samples that is required to achieve detection also varies in complexity. Because the accurate identification of biological targets within a sample typically requires culturing, PCR, or both, the time frame for sample collection, sample processing, and ultimately, target identification can be on the order of hours to days. Thus, a preliminary screening of samples would be advantageous to the first responder and would minimize the number of samples that require extensive processing in the laboratory.

The characterization of a sample as pathogenic can be accomplished using a number of different detection techniques. One method is the comparison of the volatile organic compounds (VOCs) that are

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metabolized by microorganisms[1-7] and can be detected in the headspace over samples such as bacterial cultures and spore powders. Likewise, infection of a host by bacteria may also be detectable by examining the VOCs either produced by the bacteria that are invading the host,[8] or possibly produced during the host response to the infection. Thus the classification of a sample headspace has use from both a biodefense and a biomedical standpoint. To characterize a sample as pathogenic requires a unique volatile compound or a signature, a combination of compounds present. The sample can be classified based on the presence or absence of compounds as well as comparing the relative compound abundances. The first step in developing a detector based on VOCs is to evaluate the headspace of target pathogens to determine the unique compounds or signatures. Equally important is the characterization of backgrounds that may interfere with target features.

One technique that is often used to examine the headspace of a sample is thermodesorption gas chromatography-mass spectrometry (GC-MS). The use of sorption tubes for the collection of sample headspace offers the advantage of concentrating gaseous compounds prior to separation and detection. This preconcentration step is especially important when working with trace amounts of sample. The use of GC-MS is not a novel technique for the characterization of bacterial headspace or for breath analysis. GC-MS has been utilized mainly to analyze VOCs for identifying bacteria present in buildings,[9] water treatment systems and organisms responsible for food spoilage.[10-12] The analysis of breath using GC-MS has proven useful for measuring alcohol breath content,[13,14] exposure to industrial chemicals,[15-18] monitoring human metabolism and nutrition,[19-21] and diagnosis of liver function,[22-24] intestinal complications[25] and other illnesses.[26-32]

Realizing the potential of using the volatile signatures of bacteria to enable the classification of unknown samples as pathogenic, we have developed and optimized methods to collect bacterial headspace and breath samples. Due to the constraints of many biodefense scenarios in time, cost, and personnel, we imposed a short collection time and simplified the methods and equipment required for sample collection and processing. Once our methods were optimized, we collected gas phase signatures of vegetative bacteria including potential biowarfare agents such as *Bacillus anthracis* (Ba), and *Yersinia pestis* (Yp), as well as *Bacillus thuringiensis kurstaki* (Btk), which is closely related to Ba and often used as a biowarfare simulant. We also examined sporulated bacillus species *Bacillus thuringiensis israelensis* (Bti) and *Bacillus globigii* (Bg) in various stages of washing and preparation. Simulants of dry bacterial spores including white powders such as ovalbumin, flour, baking soda, and cornstarch have been sampled to evaluate if spore powders can be distinguished from potential interferents. In addition to biowarfare-related targets we have also sampled bacteria known to cause respiratory infections, such as in the case of cystic fibrosis (CF) patients, including *Pseudomonas aeruginosa*, Methicillin resistant *Staphylococcus aureus, Burkholderia cepacia dolosa*, *Burkholderia cepacia multivorans*, and *Burkholderia cepacia cenocepacia*. Breath samples from a variety of individuals, including subjects diagnosed with CF, have been obtained and are being analyzed for the variability of chemical signatures. These background results will allow us to compare signatures of infected versus healthy individuals, and to correlate breath signatures with the headspace collected from relevant bacteria.

2. METHODS

2.1 SAMPLES

2.1.1 VEGETATIVE BACTERIA

The vegetative bacteria were obtained from the following: *Bacillus anthracis* (Sterne) from USAMRIID, *Bacillus thuringiensis kurstaki* from ATCC #33679, *Bacillus globigii* from Dugway Proving Ground, *Bacillus cereus* from ATCC #4243, and *Yersinia pestis* (KIM5 D1) from Michigan State University. *Pseudomonas aeruginosa*, Methicillin resistant *Staphylococcus aureus*, *Burkholderia cepacia dolosa*, *Burkholderia cepacia multivorans*, and *Burkholderia cepacia cenocepacia* were a provided by Children's Hospital Boston.

Stock cultures of each bacterium were grown up overnight in BHI. Bacterial lawns were formed by plating 100 µL of the stock bacterial culture onto tryptic soy agar (TSA), and were allowed to grow for approximately 18 hours at either 33 or 37 ˚C, depending on the organism. BHI and TSA were obtained from Difco.

2.1.2 BACTERIAL SPORES

Dugway Proving Grounds provided *Bacillus globigii* spores. The spores were provided as a dry, roughly milled powder, however were not refined for aerosolization and contained residual growth media. Four additional spore samples were obtained from Dugway Proving Grounds. *Bacillus thuringiensis israelensis* (Bti) and Bg spores were supplied in a highly purified, aerosolizable powder form. Bti was provided from samples processed using two different refinement methods. Small amounts of the refined powders were difficult to weigh out quantitatively, and thus the amounts used are approximately ~20 µg per sample weighted out to the best of our ability.

2.1.3 SIMULANT POWDERS

Ovalbumin was obtained from Dugway Proving Ground. Baking soda, cornstarch, bleached flour were purchased from a local food store. Amounts are provided in the text.

2.2 SAMPLE COLLECTION

2.2.1 BACTERIAL HEADSPACE OF VEGETATIVE BACTERIA, SPORES, AND SIMULANTS

Samples were weighed on a Mettler Toledo AG204 balance and then transferred to glass petri dishes (Pyrex 100 x 15 mm) allowed to equilibrate at either 37 ºC (to simulate body temperature, and growth conditions) or 100 ºC (for increased off gassing of compounds). Headspace was collected by enclosing the petri dish with sample in one of two chambers custom made of glass with the dimensions of 6 ½" h x 12" diam. and 2 ¾" h x 5 ½" diam. which will be referred to as chamber A and chamber B, respectively. Chromatograms that are presented herein are compared only when taken under the same conditions.

Chamber A was comprised of the body of a dessicator placed upside down on top of a passivated metal plate. Inlet and outlet valves controlled the airflow through the chamber. The total volume inside the chamber was approximately 6 L. After samples were placed inside the chamber, the chamber was pressurized with compressed air (Airgas) to one psi. The sample remained inside the sealed chamber long enough for the VOCs to come into equilibrium with the chamber volume, typically two and a half hours. The air in the chamber was pulled through a Tenax TA thermodesorption tube using an evacuated SUMMA canister.

Chamber B was designed as an upgrade to the sample collection process. This chamber was constructed entirely from glass, including the cover as well as the gas inlets. An o-ring sealed the cover to the base, and is held in place with three clamps. Room air was used as the carrier gas through the sample chambers, and was first filtered with a Whatman Vacu-Guard filter, and then scrubbed with an Aeronex Gate Keeper gas purifier. Tygon tubing was used for gas flow into and out of the sample chambers, and Whitey teflon valves permit a closed environment to be achieved if desired. The rate of airflow through the sample chambers was controlled by a Gerstel GS1 Gas Sampler, which directs air through sorption tubes for concentration. Tenax TA sorption tubes (Gerstel, US) were used for all sample collections. Each sample is collected for 10 minutes at a rate of 0.5 L/min. Prior to sample collection, the Tenax TA tubes are conditioned for one hour at 250 ºC, while being purged with ultra high purity nitrogen gas (Airgas).

2.2.2 BREATH COLLECTION

Breath samples were collected in 8.1 L Tedlar bags, outfitted with ON/OFF valves. A short piece of a drinking straw was fitted on the ON/OFF valve to make sample collection easier. Subjects were instructed to hold their breath for approximately 10 seconds before expiring completely into the bag. The valve was closed between each breath. The samples were processed by concentrating the air sample onto Tenax TA tubes using the Gerstel Gas Sampler, sampling at a rate of 0.60 L/min for approximately 15

minutes. The Tedlar bags were connected to the gas sampler with a piece of 6-inch Nafion tubing to remove moisture from the samples. The sampling rates were chosen in order to remove close to 100% of the breath moisture.

2.3 SAMPLE PROCESSING

All Tenax tubes are loaded onto a Gerstel TDS-A cartridge, which interfaces with the GC-MS system (Agilent Technologies 6890N/5973). Samples were concentrated using a cooled injection system (Gerstel CIS 4) set to –150 ˚C. The temperature of the CIS then increases at a rate of 60 ºC/min to 250 ˚C to transfer sample onto the GC column (DB-624 capillary column; 25.0 m long, 0.2 mm I.D., 1.12 µm film thickness). The GC temperature program was set to start initially at 40 ˚C for 1 min, and then increasing at a rate of 5 ˚C/min, to 150 ˚C. The MSD was scanned from 35 – 500 amu with 1.63 scans/second. Three samples were taken per scan.

2.4 DATA ANALYSIS

All peak assignments were performed using the NIST 2002 compound library.

2.5 METHOD VALIDATION

Method reproducibility has been confirmed by repeating samples on different days. Samples are also run in triplicate with the headspace of the relevant background collected in triplicate prior to each sample. Collection of background spectra is imperative due to the headspace chamber method we are currently using. The backgrounds take into account any changes in the lab environment, including source air, and some off gassing from chamber components. Designing inexpensive disposable chambers that will eliminate any sample carryover, and minimize chamber-related compounds is currently enhancing the chamber method.

3. RESULTS AND DISCUSSION

3.1 VEGETATIVE BACTERIA

Vegetative bacteria have been previously shown to possess unique signatures when the headspace is collected and analyzed using GC-MS.[1-7] The primary focus of studies published to date has been on the signatures produced by organisms responsible for food spoilage and contamination.[10-12] Little or no data is available on the bacterial species that could potentially be used as biowarfare agents or simulants. If biowarfare agents such as *B. anthracis* and *Y. pestis* present unique signatures relative to environmental backgrounds (room air, dry filter unit collections), simulants (ovalbumin, white powders), and related bacterial species (*B. thuringiensis kurstaki)*, simple methods could be developed to enable rapid, non-invasive screening of samples for the presence of such pathogens. As a proof of concept that these bacteria have distinguishable chromatograms, we grew cultures of each bacterium on TSA. Cultures grown for approximately 18 hours resulted in a lawn of bacteria that were then transferred into Chamber A. The headspace of each bacterial species was then collected. The chromatograms shown in Figure 1 compare the signatures of the three pathogens versus the headspace of TSA alone.

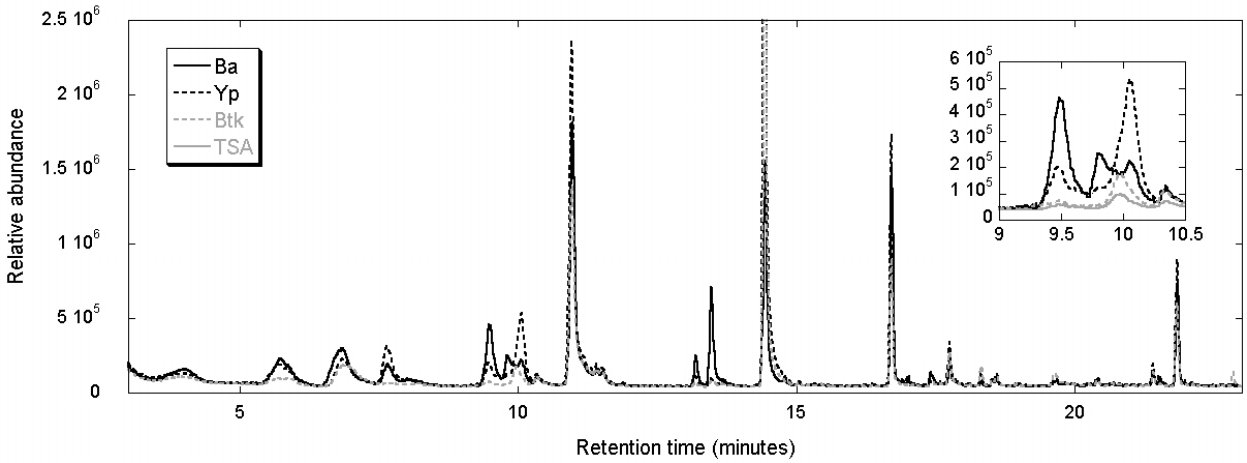

FIGURE 1. Headspace chromatograms of 18-hour cultures of *B. anthracis* (Ba), *Y. pestis* (Yp), and *B. thuringiensis kurstaki* (Btk) on TSA. Headspace of each sample was collected for 2.5 hours in Chamber A. The inset shows the region of 9-10.5 minutes in more detail.

Many of the same compounds are observed in all four spectra. This is not surprising as each of the bacteria has been grown on the same medium and thus compounds from the medium itself will be included in each chromatogram. In addition, metabolism of the bacteria is also dependant on the type of medium available and many of the same metabolites will likely be produced. However, although each of the bacteria was grown on TSA, both the presence and relative abundance of several compounds appear to be species related. An example of this is the incidence of ethyl benzene and 1,3-dimethyl benzene in the chromatogram of Ba. These compounds can be found at retention times of 13.2 and 13.5 minutes, respectively, in Figure 1. The production of 3-methyl-2-pentanone (t = 10.0 min) appears to be more prominent in cultures of Yp, and toluene is only observed from the culture of Btk (t = 9.9 min). One compound that appears common to all four bacteria at different levels, though rarely seen in samples not containing bacteria, is the reduced sulfur compound dimethyl disulfide (t = 9.4 min). The presence of reduced sulfur could be one simple indicator that a sample is bacterial in nature. Many improvements on the sample collection technique were made based on results of the first spectra. These initial, promising results prompted further studies.

In addition to our interest in bacteria that could potentially be used as biological threat agents, we have also begun characterizing the volatile metabolites produced by bacteria known to cause respiratory infections in humans. From a biodefense standpoint, it would be advantageous to be able to screen individuals thought to have been exposed during a bioagent release by collecting a non-invasive breath sample. From a clinical standpoint, the pre-symptomatic diagnosis of an infection could speed up or enable treatment of many diseases.

It has long been surmised that the breath of an individual contains valuable information about their health status. Several examples that support this hypothesis include the detection of increased levels of acetone in the breath of diabetics,[33] and pentane in the breath as a result of liver cirrhosis.[34] Recently, it has also been shown that cancer can be detected by the presence of alkanes and monomethylated alkanes.[29] Many studies have focused on changes in the production of nitric oxide (NO) as an indicator of a health condition.[35] This is difficult to monitor due to the multitude of physiological influences on NO production, as well as the relatively small changes in NO that must be accurately detected. We have begun studying the signatures of pathogens that cause respiratory infections based on the supposition that bacteria residing in the lungs will produce a headspace much like that produced in vitro. Alternatively, infection could be monitored by examining the compounds produced as a result of systemic effects. These compounds that are present in the circulatory or immune systems are in equilibrium with the alveolar air in the lungs and thus should be measurable by breath. Of course, the differences in volatile byproducts of bacteria grown in vitro versus in vivo will be important to recognize and characterize.

We have focused on the signatures of the opportunistic pathogens in cystic fibrosis (CF) patients. The CF population presents a helpful case, as individuals are monitored by healthcare professionals on a regular basis. This provides a means to examine the breath of individuals at varying stages of infection, and throughout antibiotic treatments. Additionally, the species of bacteria that commonly cause infection in CF patients are well known and do not generally cause infection in the general public. These bacteria include *Pseudomonas aeruginosa* (Pa), Methicillin resistant *Staphylococcus aureus* (MRSA), and several subspecies of *Burkholderia cepacia*. Figure 2 displays the gas chromatograms of two representative bacteria (*Pseudomonas aeruginosa* and *Burkholderia cepacia cenocepacia*) that were originally cultured from CF patients, and were grown overnight on TSA. Samples were collected for 10 minutes in Chamber B. The decrease in sampling time was the result of method optimization toward simpler, more rapid sample collection. Again, distinct signatures are representative of each bacterium grown on the same agar. To make this point, *Burkholderia cepacia cenocepacia* (Bcc) and *Pseudomonas aeruginosa* (Pa) are plotted in the same graph. The chromatogram of Bcc shows a large peak at t = 6.6 min and a number of smaller peaks at t = 10.2, 11.2, and 11.8 min. These peaks have been identified as isobutyronitrile, 2-methyl-propanal oxime, pyrrole, and 1-nitro-butane, respectively. Bcc also displays a large abundance of the compound dimethyl disulfide at 9.3 min. Pa has unique peaks including acetamide and propanamide at t = 13.2 and 15.8 min. A number of compounds which are not listed are either common between bacteria or attributed to the background. Spectra of *Burkholderia cepacia dolosa*, *Burkholderia cepacia multivorans,* and Methicillin resistant *Staphylococcus aureus* were also measured but are not shown here.

Breath samples have been collected from two individuals diagnosed with CF, to date. One subject was sampled several times in good health and another was sampled amidst an acute respiratory infection just before hospitalization. A recently established collaboration is providing the opportunity to sample patients diagnosed with CF as well as other common respiratory illnesses. Breath sample results and discussion can be found in Section 3.3.

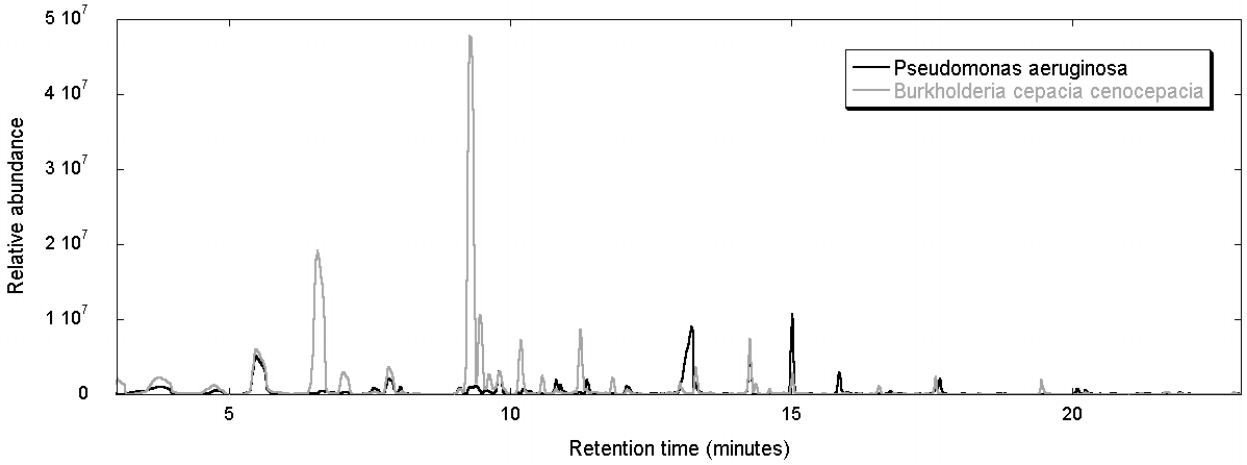

FIGURE 2. Chromatograms of *Burkholderia cepacia cenocepacia* and *Pseudomonas aeruginosa* grown on TSA in Chamber B. Headspace was sampled for 10 minutes.

It is recognized that the VOCs produced by the bacteria will not only be species dependant, but also dependant on the growth conditions, including the growth media used and the phase of bacterial growth at the time of headspace collection. This correlation could be used to an advantage if it was necessary to determine pathogen viability. To this end, we can detect changes in the VOCs produced during growth of bacteria on agar. It is possible to observe changes in volatile metabolites long before the ability to visualize the formation of colonies. Thus, if an unknown sample was grown on a predetermined media, the sample headspace can be monitored for the presence of peaks expected from a particular pathogen on that media.

We looked at the growth of both Pa and Bti on TSA. Headspace collections of Pa were taken starting at 1.5 hours and then continued at 1.5-hour intervals. (Only every other sample is shown for spectral clarity.) Within the first 1.5 hours the decrease of 3-methyl-butanal at 6.8 min is already evident. Between 4.5 and 7.5 hours, a large increase in compounds is observed in the headspace. Examples of this include the compounds 2-butanone, 2-pentanone, 3-methyl-3-buten-1-ol and 3-methyl-1-butanol eluting at retention times of 5.5, 7.8 and 9.7 min, respectively. In the case of the peak at 9.7 minutes, the increase is sharp until 7.5 hours and then there is little further change. On the other hand, peaks at 5.5 and 7.8 minutes continue to increase in abundance for the duration of sampling.

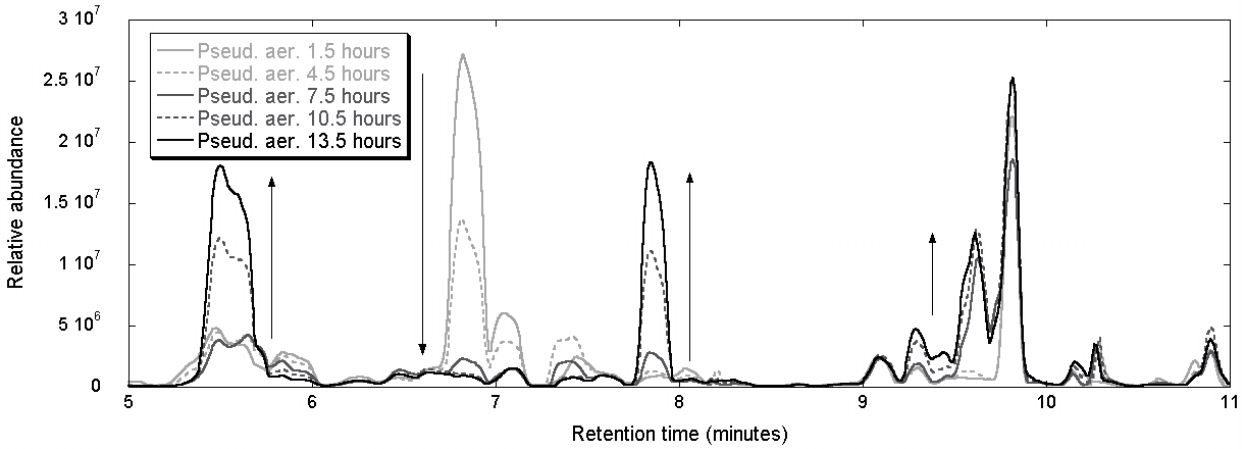

FIGURE 3. Growth of *Pseudomonas aeruginosa* on TSA.

A second example that was explored is the germination and growth of viable Bti spores on TSA, shown in Figure 4. In this case, approximately 20 mg of refined Bti spores were plated onto TSA and placed in Chamber B where headspace was drawn from the chamber for 2.5-hour intervals. In this case as well, there is an obvious change in several compound abundances from one collection to the next. It is likely that the rapid decrease in the compound 3-methyl-butanal at ~7.0 min is due to a combination of two factors: the metabolism (and thus the breakdown) of compounds present in the media and by dilution. On the other hand, the increase in the abundance of dimethyl disulfide (DMDS) at 9.4 min and 2,4-dithiapentane at 14.5 min can only be due to production as a result of bacterial metabolic processes. Further experiments have shown that the DMDS is present at detectable levels in as few as 45 minutes. Detection at even shorter times is likely possible by optimizing the method of detection. Furthermore, the addition of compounds such as L-alanine to spore solutions can stimulate germination and would thus enable a more rapid means of detection and viability testing. Experiments to correlate compound production with the quantity of bacteria present and the phase of bacterial growth are underway.

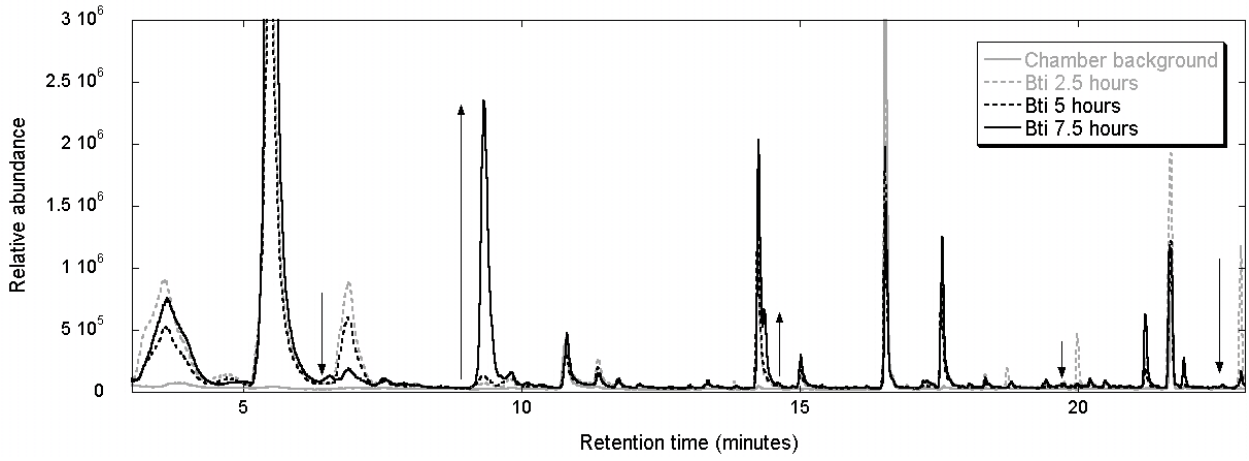

FIGURE 4. Germination of *B. thuringiensis israelensis* (Bti) spores on TSA.

3.2 BACTERIAL SPORES

Sporulated bacteria present a challenge for detection. The difficulty lies in achieving adequate detection sensitivity. In the case of spores, the organisms are dormant and thus are not continuously producing volatile metabolic products throughout the collection period. In many cases spores are "cleaned," where successive washings in DI water, surfactant solutions, or separation with Renografin gradients are used to remove the media and residual cellular debris. Likewise, spores that are used as biowarfare agents may be processed further to make them aerosolizable. These treatments can vary, and thus the chemicals off gassed from the spores will differ as well. Therefore, detection relies on the collection of volatile compounds that remain on the bacterial spores after being cleaned, dried and processed into powders. The following data demonstrate that even highly purified spores may be distinguishable based on the bacterial species and/or chemical treatments. As mentioned previously, germination of the spores can also be monitored as an indication of sample viability and may offer more species-specific information.

Maintaining a short 10-minute headspace collection time as well as the 37 °C chamber temperature, we measured the gas chromatogram of 1 mg dry Bg spores versus background in Chamber B (Figure 5). A unique signature from the Bg spores is clearly observable over the background. The compounds in greatest abundance are acetone (t = 3.7 min.) and 2-methyl-2-propenoic acid methyl ester (t = 8.1 min.). In addition, peaks that are found in the chromatogram of Bg that are not observed in the background also include, for example, 1-butanol, 4-methyl-3-pentene-2-one, 1,3-dimethyl-benzene and phenol, eluting at 7.4, 11.4, 13.3, and 20.2 minutes, respectively. In this case, the sample of Bg spores likely contained residual sporulation medium, and therefore the medium is probably the origin of many compounds observed. Unfortunately we do not have the specific formulation for the Bg sporulation media or information about the processing conditions. Correlations of the observed compounds with compounds expected as a result of the spore preparation could not be made with this sample.

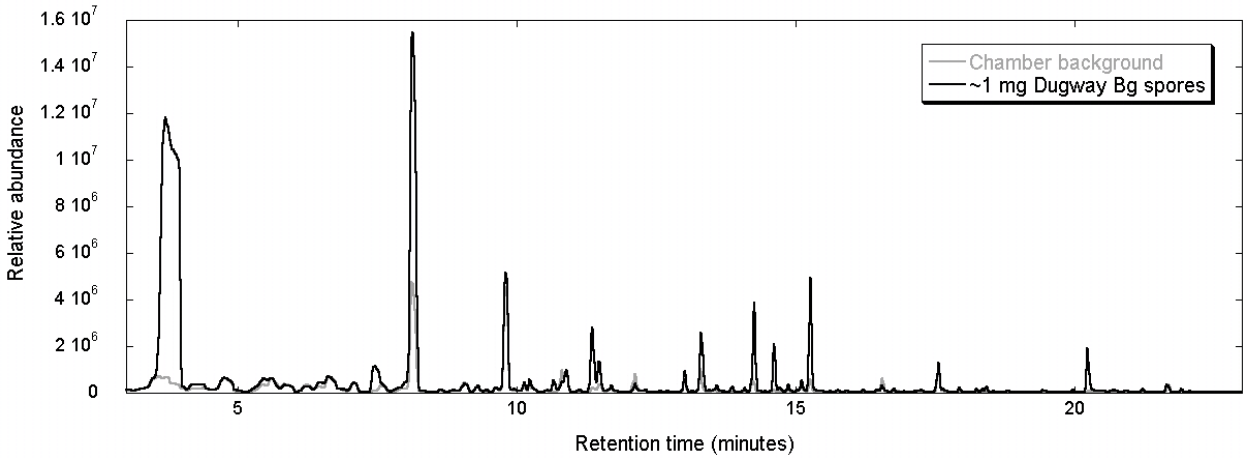

FIGURE 5. Chromatogram of dry *Bacillus globigii* spores versus background in Chamber B. Samples were collected for 10 minutes on a glass petri dish.

To extend this study we also examined Bg and Bti spores that have not only been dried into a powder, but also refined using two different processes. The chromatograms of the same Bti spores with varied preparation are displayed in Figures 6a and 6b. In this case, compounds that are observed in only one of the two chromatograms can be attributed to the preparation method. The compounds in the chromatogram of Bti Prep #1, eluting at 3.7, 5.4, 9.7 and 11.4 minutes, are primarily ketones. This is in comparison to the peaks at 7.5 and 15.2 minutes, which are found only in the Bti spore sample that was refined using Prep #2. The graphs in Figures 6c and 6d exemplify the differences in bacterial species (Bti versus Bg) when prepared using the same method (Prep #2). Notably, the peak at 15.2 minutes, observed in Figure 6d is not observed in the spectrum of Bg that has undergone preparation #1. One particular peak, 2-methyl-2-propenoic acid methyl ester eluting at 8.1 min is specific to Bg spores and has been seen in Bg spores (Figure 5 above) that have not undergone the rigorous cleaning and refinement processes. This suggests that spore sub-species may be discriminated when examining the differences in spectra between Bacillus spores. The compounds 2-methyl-propanol, 4-cyanohexene and phenol shown at 6.4, 10.9 and 20.2 minutes, respectively, are observed in all spectra 6a-d and may be common to the Bacillus species in general. It is certainly much more difficult to detect changes in spore species when the spores are highly purified and cleaned, however we are currently exploring methods to improve the sensitivity, such as increasing the sample temperature during headspace collection.

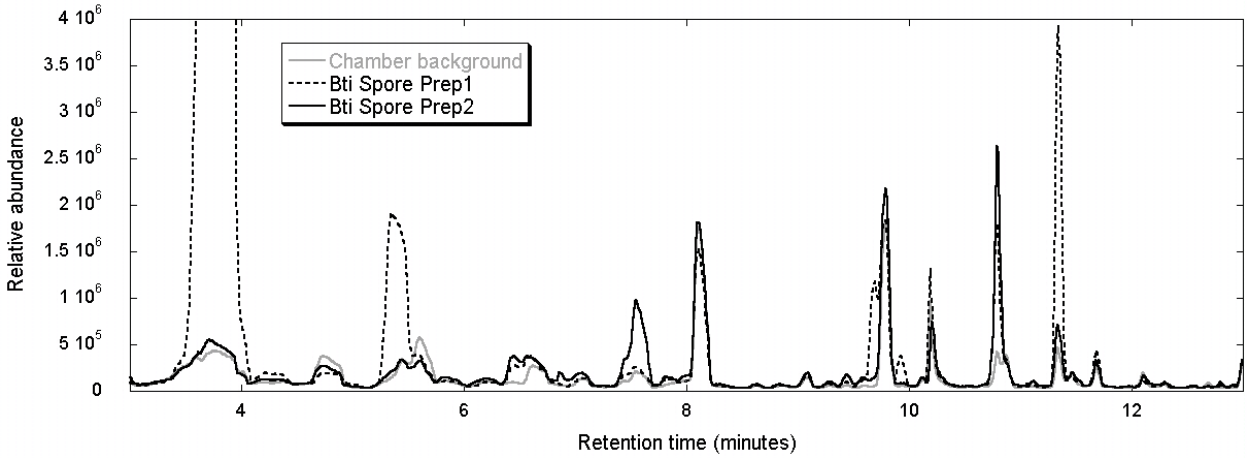

FIGURE 6a. Dry *B. thuringiensis israelensis* spores refined using two different methods. Samples were collected for 10 minutes in Chamber B. Compound retention time 3-13 minutes.

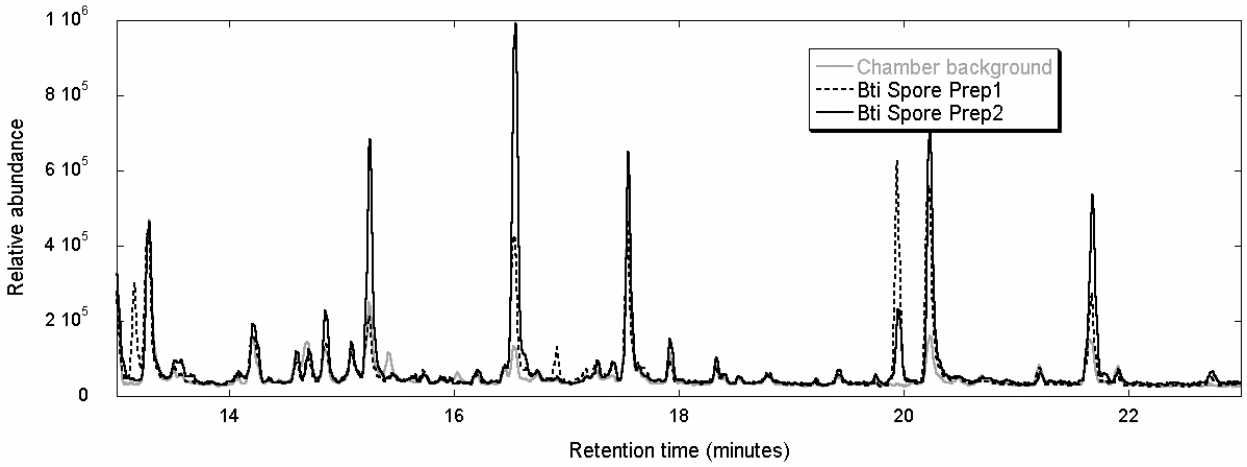

FIGURE 6b. Dry *B. thuringiensis israelensis* spores refined using two different methods. Samples were collected for 10 minutes in Chamber B. Compound retention time 13-23 minutes.

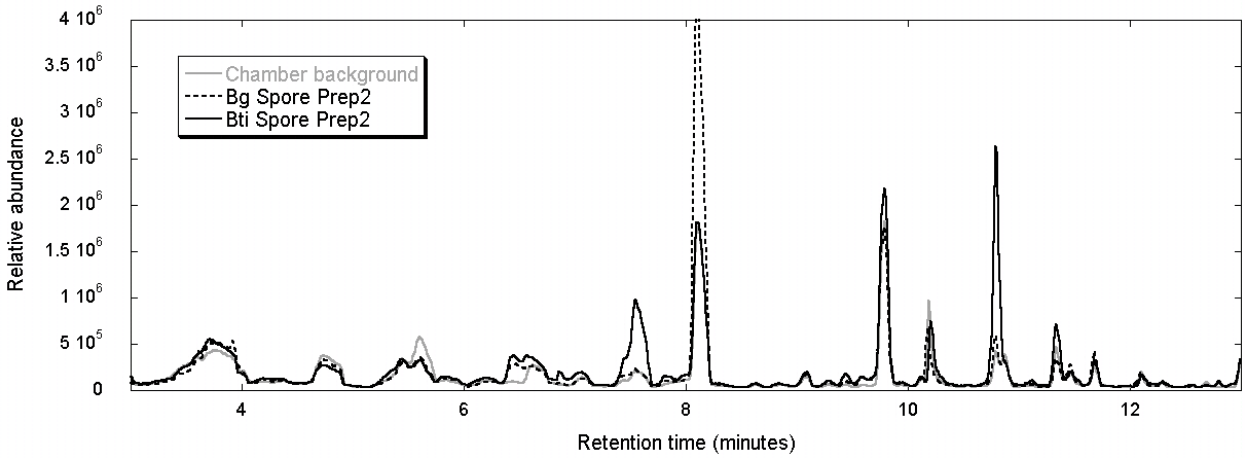

FIGURE 6c. Dry *B. thuringiensis israelensis* and *B. globigii* spores refined using the same method. Samples were collected for 10 minutes in Chamber B. Compound retention time 3-13 minutes.

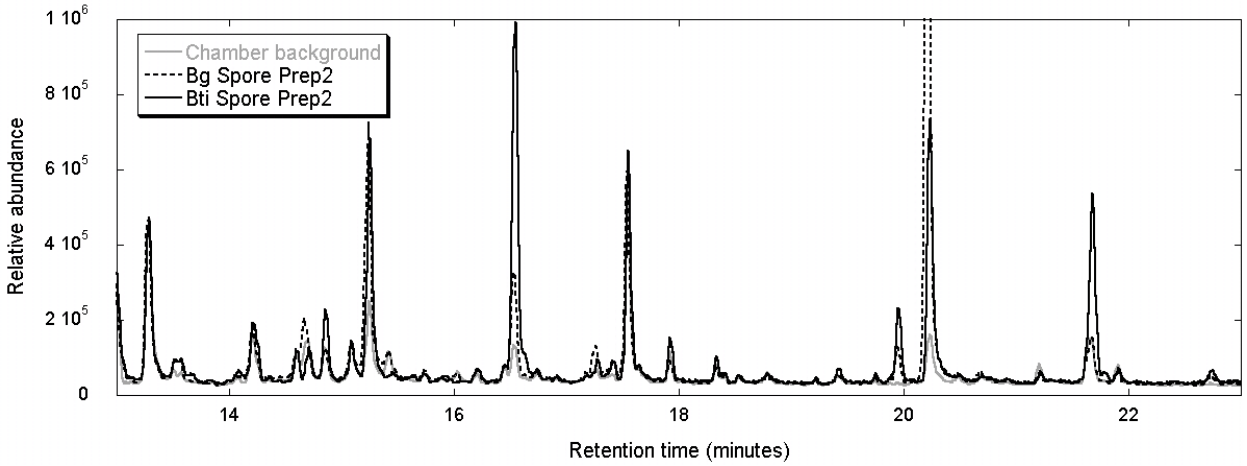

FIGURE 6d. Dry *B. thuringiensis israelensis* and *B. globigii* spores refined using the same method. Samples were collected for 10 minutes in Chamber B. Compound retention time 13-23 minutes.

Another challenge that we wanted to undertake was the discrimination of spores versus simulants, such as non-biological powders or background interferents. The spectra of Bg spores versus bleached flour and non-bleached powders (i.e. ovalbumin, baking soda, corn starch) are shown in Figure 7. The most noticeable aspect of the chromatograms is the large increase in number and abundance of compounds that come from the sample of Bg spores even though the amounts of sample are the same. Several peaks that are notably different in the spectrum of Bg include 4-methyl-4-penten-2-one (t = 9.9), 2,4-dimethyl-pyridine (t = 15.8), 2,4,6-trimethyl-pyridine (t = 17.6), 2-ethyl-4,6-dimethyl-pyridine (t = 20.1), 2,3,-dimethyl-2-cyclopenten-1-one (t = 20.9), and 1-methyl-2-pyrrolidinone (t = 21.26). In this case the amounts of spores and powders sampled were on the order of 1 gram. Additional measurements were taken at later dates with much smaller amounts (< 20 μg) and the difference between spores and powders was less prominent yet still distinguishable.

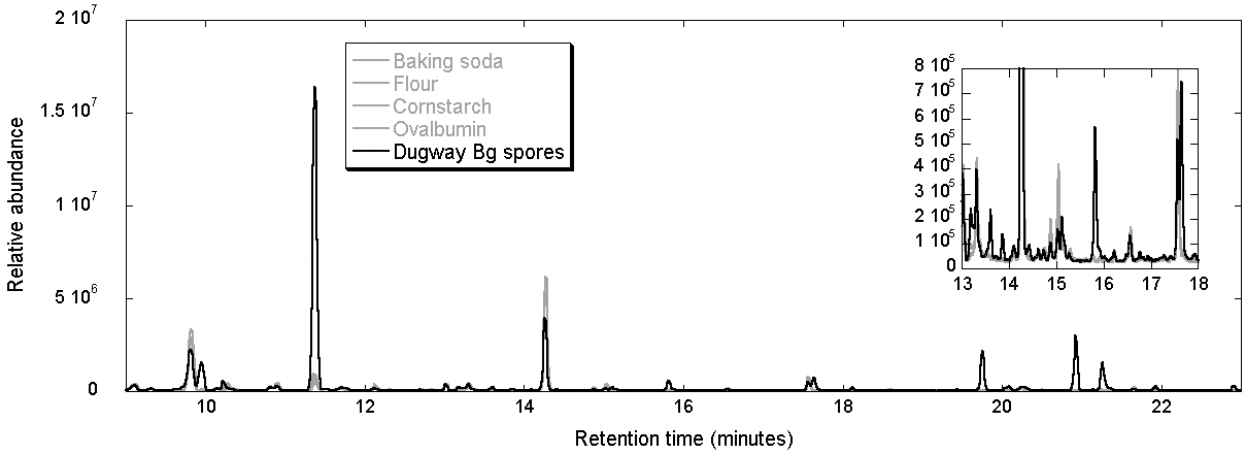

FIGURE 7. Dry *B. globigii* spores versus dry powders. Approximately 1 gram of each sample in a glass petri dish, 10-minute sample collection using Chamber A. The inset shows the 13-18 minute region in more detail.

3.3 BREATH SAMPLES

The optimization of methods to collect breath samples was also undertaken in parallel to the collection of bacterial headspace signatures. The use of thermodesorption tubes to concentrate VOCs allows for the sampling of large volumes of air while concentrating the important signatures. This technique is well suited for the collection of breath samples since the majority of each breath exhalation is composed of dead space air that is not in equilibrium with the circulatory system. Sorption tubes enable the collection and concentration of the vital compounds in breath without the use of complex instrumentation to separate the signature-rich alveolar air from the less revealing dead space. Another important optimization for the collection and processing of breath samples is the use of Nafion tubing to remove moisture prior to the concentration of VOCs on the sorption tube. An example of a spectrum resulting from this collection scheme is shown in Figure 8. In this instance, the breath of a healthy individual diagnosed with CF was collected and compared to the same volume of background air collected in the same place the air sample was collected. It is not difficult to see that the person's breath contains a number of compounds that are not present in room air itself. Several compounds that are distinctive include allyl methyl sulfide (7.4 min), (Z)-1-(methylthio)-1-propene (8.0 min), and (E)-1-(methylthio)-1-propene (8.7 min).

A large number of breath samples have been collected to examine the variability of breath VOCs among different subjects, and also for the same subject under different conditions (i.e time of day). Ultimately, we aim to be able to classify an individual as having a bacterial infection or having been

exposed to a particular pathogen without the need to control for environmental factors. The discrimination would therefore be based on the presence of unique compounds or signatures that are not normally observed in breath.

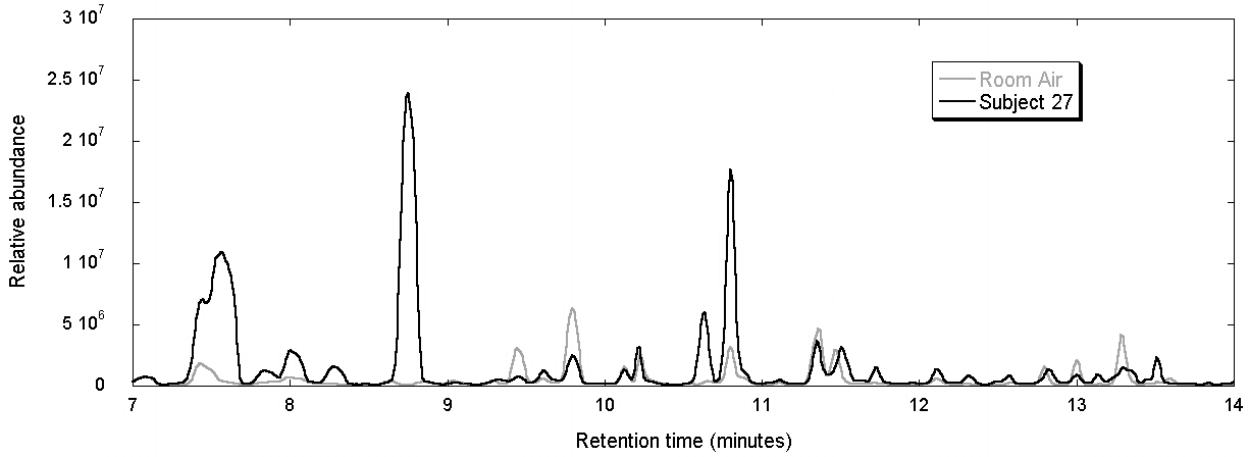

FIGURE 8. Gas chromatograms showing the breath of a "healthy" CF patient (Subject 27) versus room air concentrated on a Tenax TA sorbtion tube. The same volume of sample was collected in both cases.

The breath chromatograms of three individuals are shown in Figure 9. While many compounds are similar in each of the three subject samples, as well in many of the other samples we have collected, there are several compounds that do not appear ubiquitous to breath in general. Interestingly, Subject #45 is also diagnosed with CF and shows many of the same compounds as #27, albeit in much lower concentrations. In comparison to the peaks listed for both subject #27 and 45, subject #14, who does not have CF, does not present the unique (methylthio)propene compounds but rather exhibits a large increase in 2-hexanone (9.4) and 4-heptanone (13.8). In order to prove that the compounds are reproducible and are not dependant on a coincidental environmental factor, it should be noted that the breath of patient #45 was taken at a different location then #27, and as shown in Figure 10, the breath of subject #27 was collected on multiple days and the (methylthio)propene compounds appear to be persistent. In general we have observed that the relevant compounds in the breath of a single individual do not vary appreciably with time. Current efforts are focused on assessing the dependability of breath signatures as health indicators, as well as developing algorithms to determine if signatures can be statistically correlated to the health of an individual. A large increase of data is expected to become available for analysis throughout the upcoming cold and flu season.

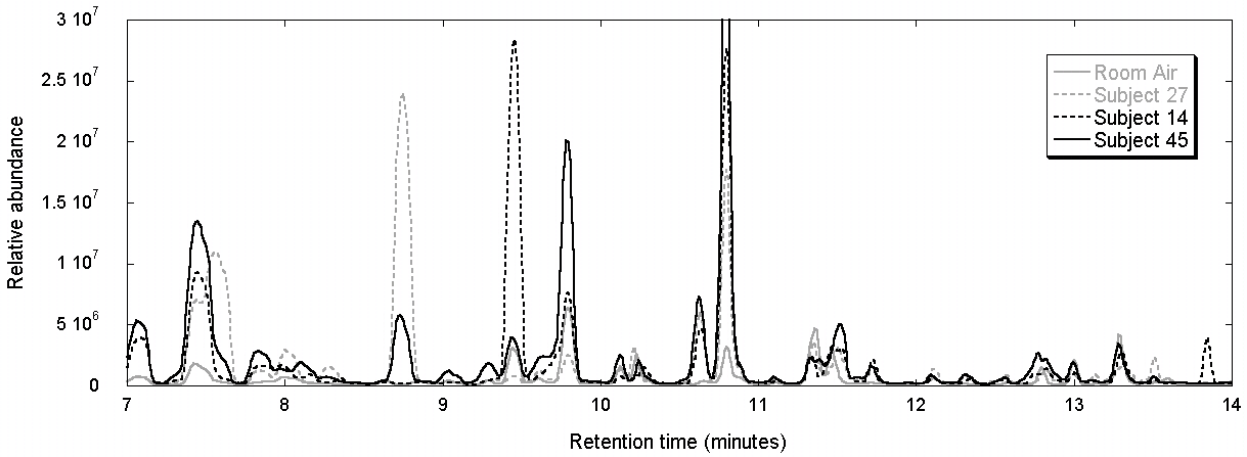

FIGURE 9. Gas chromatograms comparing the breath samples of three subjects versus room air. Subjects 27 and 45 have been diagnosed with CF; Subject 14 has a non-respiratory bacterial infection.

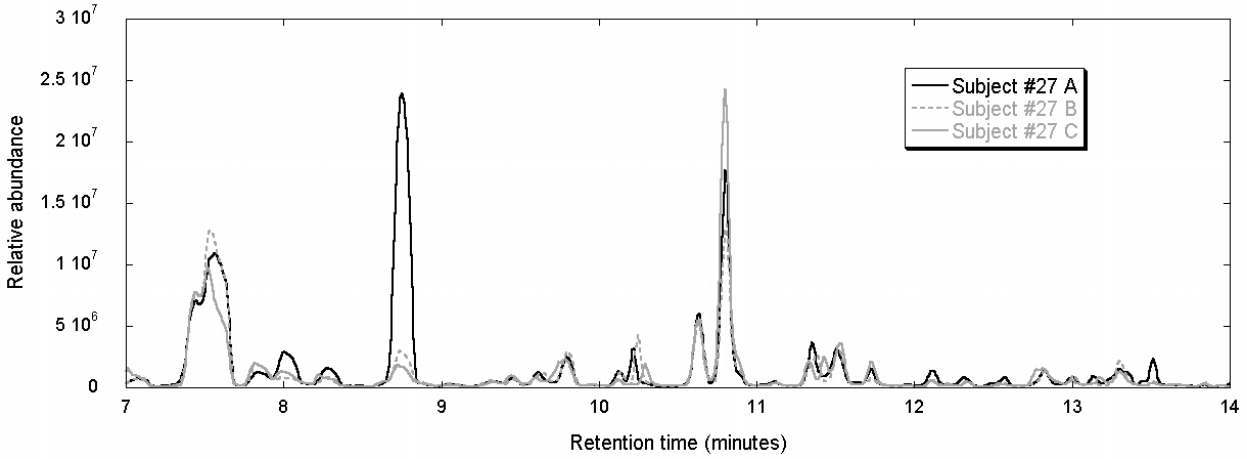

FIGURE 10. Gas chromatograms comparing the three breath samples of Subject 27 taken on different days.

3.4 STATISTICAL ANALYSIS

Qualitative assessment of the gas chromatographs of various spores and simulant powders has motivated the development of statistical methods to discriminate between biological spores and other materials. Such methods will ultimately be the basis for an automated deployable system to characterize suspicious powders or to perform early diagnosis based on breath sampling. In addition, the statistical approach provides insights that are not available through qualitative analysis alone. Statistical analysis can exploit subtle patterns and help to identify combinations of compounds that are indicative of spores or spore preparations but which are still present to lesser degrees in other materials. This analysis can also account for variations between samples of a given spore or powder and can provide a quantitative measure of significance for the findings.

The problem is set up as a two-class discrimination problem. Class ***S*** represents headspace measurements from spores while class ***NS*** is a negative control that may contain headspace measurements from simulant powders or from an empty chamber. Several sets of GC-MS measurements have been

collected for each of the spores and spore preparations (Bg and Bti) as well as for the simulant powders (ovalbumin, flour, baking soda, and corn starch) and empty chambers. Each set of measurements is assigned a truth label of either ***S*** or ***NS***, as appropriate.

The full set of measurements from a single sample is a very high-dimensional integer vector, consisting of over two thousand mass spectra, each with an eight hundred mass-to-charge histogram bin resolution. Considered as a whole, these data constitute an integer vector having over a million dimensions. In order to reduce the number of dimensions, an automatic library search is employed to identify eluted compounds based on the GC-MS data. Several thousand candidate eluates have been identified, and a new feature vector for each sample is constructed by associating a combined quality and abundance score with each of the potential eluates. This approach to reducing dimensions has the additional benefit of providing a feature vector with a clear physical interpretation in terms of constituent compounds.

A two-class discriminator is designed by training a multiple linear regression algorithm on the derived feature vectors and associated truth labels. Performance is assessed by cross-validation, where the discriminator is trained on subsets of the available data and tested on the data not used for training. Current results are preliminary, but they suggest a capability to accurately discriminate spores from simulant powders and/or empty chambers with an accuracy of 80-90% or greater. It is also possible to identify the sets of compounds that drive discrimination; information that could eventually be used to build more dedicated sampling systems. The analysis techniques will be further refined and more definitive results will be obtained as the database of measurements grows to be more statistically significant. More sophisticated algorithms than linear regression will also be evaluated if the data suggest that this would be appropriate.

4. CONCLUSIONS AND FUTURE DIRECTIONS

The discrimination between bacterial species and the detection of pathogens in the presence of common backgrounds and interferents has been proven possible. Scenarios for which such discrimination is useful include the pre-symptomatic diagnosis of disease by monitoring breath, the classification of samples (liquid and dry) as biological and/or viable, species discrimination of vegetative and sporulated bacteria, and information about the specific type of preparation used to process and refine biological samples such as spore powders. To accomplish this, methods for the collection and concentration of bacterial headspace as well as the concentration of breath VOCs, have been developed and optimized. The methods used herein have focused on maintaining a short sampling time and simple, reproducible procedures suited for both clinical and biodefense applications. To date, the methods we have developed have been geared toward extracting the most information possible from the headspace of the bacterial samples. Depending on the specific application, the sample collection and processing times can be shortened and the detection made increasingly sensitive. To this end, we are focusing efforts on determining the compounds critical for discrimination and developing the algorithms to make that distinction. In addition, disposable chambers for automated sampling are being designed and constructed to increase the limits of detection of bacterial targets in the presence of interfering backgrounds, as well as to progress toward a field-portable screening device.

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